

# Heart-Specific Immune Responses in an Animal Model of Autoimmune-Related Myocarditis Mitigated by an Immunoproteasome Inhibitor and Genetic Ablation

**BACKGROUND:** Immune checkpoint inhibitor (ICI) therapy is often accompanied by immune-related pathology, with an increasing occurrence of high-risk ICI-related myocarditis. Understanding the mechanisms involved in this side effect could enable the development of management strategies. In mouse models, immune checkpoints, such as PD-1 (programmed cell death protein 1), control the threshold of self-antigen responses directed against cardiac TnI (troponin I). We aimed to identify how the immunoproteasome, the main proteolytic machinery in immune cells harboring 3 distinct protease activities in the LMP2 (low-molecular-weight protein 2), LMP7 (low-molecular-weight protein 7), and MECL1 (multicatalytic endopeptidase complex subunit 1) subunit, affects TnI-directed autoimmune pathology of the heart.

**METHODS:** TnI-directed autoimmune myocarditis (TnI-AM), a CD4<sup>+</sup> T-cell-mediated disease, was induced in mice lacking all 3 immunoproteasome subunits (triple-*ip*<sup>-/-</sup>) or lacking either the gene encoding LMP2 and LMP7 by immunization with a cardiac TnI peptide. Alternatively, before induction of TnI-AM or after establishment of autoimmune myocarditis, mice were treated with the immunoproteasome inhibitor ONX 0914. Immune parameters defining heart-specific autoimmunity were investigated in experimental TnI-AM and in 2 cases of ICI-related myocarditis.

**RESULTS:** All immunoproteasome-deficient strains showed mitigated autoimmune-related cardiac pathology with less inflammation, lower proinflammatory and chemotactic cytokines, less interleukin-17 production, and reduced fibrosis formation. Protection from TnI-directed autoimmune heart pathology with improved cardiac function in LMP7<sup>-/-</sup> mice involved a changed balance between effector and regulatory CD4<sup>+</sup> T cells in the spleen, with CD4<sup>+</sup> T cells from LMP7<sup>-/-</sup> mice showing a higher expression of inhibitory PD-1 molecules. Blocked immunoproteasome proteolysis, by treatment of TLR2 (Toll-like receptor 2)-engaged and TLR7 (Toll-like receptor 7)/TLR8 (Toll-like receptor 8)-engaged CD14<sup>+</sup> monocytes with ONX 0914, diminished proinflammatory cytokine responses, thereby reducing the boost for the expansion of self-reactive CD4<sup>+</sup> T cells. Correspondingly, in mice, ONX 0914 treatment reversed cardiac autoimmune pathology, preventing the induction and progression of TnI-AM when self-reactive CD4<sup>+</sup> T cells were primed. The autoimmune signature during experimental TnI-AM, with high immunoproteasome expression, immunoglobulin G deposition, interleukin-17 production in heart tissue, and TnI-directed humoral autoimmune responses, was also present in 2 cases of ICI-related myocarditis, demonstrating the activation of heart-specific autoimmune reactions by ICI therapy.

**CONCLUSIONS:** By reversing heart-specific autoimmune responses, immunoproteasome inhibitors applied to a mouse model demonstrate their potential to aid in the management of autoimmune myocarditis in humans, possibly including patients with ICI-related heart-specific autoimmunity.

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## Clinical Perspective

### What Is New?

- In 2 cases of immune checkpoint inhibitor–related myocarditis, evidence for a cardiac Th17 immunophenotype, deposition of immunoglobulin G around injured cardiomyocytes, and immunogenicity against troponin I reflect heart-directed autoimmunity.
- The immunoproteasome, a multicatalytic protease known to induce a Th17 immunophenotype with disease-exacerbating potential in autoimmune myocarditis, is active in 2 cases of immune checkpoint inhibitor–related myocarditis and induces cardiac inflammation in experimental Tnl (troponin I)–induced myocarditis.
- Blockade of immunoproteasome function in Tnl-induced myocarditis decorates CD4<sup>+</sup> T cells with inhibitory checkpoint molecules, suppresses pro-inflammatory cytokine production by monocytes, and elevates regulatory T-cell responses, thereby reducing inflammatory heart tissue damage and improving cardiac function.

### What Are the Clinical Implications?

- In PD-1 (programmed cell death protein 1)–related and PD-L1 (programmed cell death ligand 1)–related immune checkpoint inhibitor–related myocarditis, a high-risk immune-related side effect of immune checkpoint inhibitor cancer immunotherapy, this study demonstrates heart-specific autoimmune response.
- By mitigating cytokine production, blocking the boosting of effector T cells and delivering inhibitory signals that increase T-cell self-tolerance, immunoproteasome inhibitors might aid in the management of myocarditis with evidence of heart-specific autoimmune responses.

The antigen-recognition signaling system is equipped with immune checkpoint molecules that regulate the threshold of antigen responses and prevent overactivation.<sup>1</sup> Immune checkpoint inhibitors (ICIs) targeting checkpoint molecules such as PD-1 (programmed cell death protein 1), PD-L1 (programmed cell death ligand 1), and CTLA-4 (cytotoxic T-lymphocyte–associated protein 4) have revolutionized treatment strategies for a range of solid and hematologic malignancies. Unleashing tumor-specific T cells in patients with cancer comes at the price of general activation of T cells, often resulting in autoimmunity.<sup>2</sup> Autoimmune-related disease as a result of ICI therapy often affects the skin, colon, lung, endocrine system, or renal system.<sup>3</sup> Myocarditis is a less common but often fulminant and severe side effect of ICI therapy that may be difficult to diagnose.<sup>4</sup> The majority of patients

diagnosed with ICI-related myocarditis had abnormal ECG findings and elevated troponin levels, but most patients had a normal ejection fraction.<sup>4–6</sup> Beyond case studies, over time, a substantial increase in the incidence of ICI-related myocarditis has been documented in safety databases, and its high mortality rate has been highlighted recently.<sup>7</sup>

Preclinical mouse models have implicated the indispensable role of the PD-1/PD-L1 pathway in peripheral tolerance of autoreactive T cells targeting cardiac autoantigens. Genetic deletion of PD-L1/PD-L2 (programmed cell death ligand 2), as well as treatment with anti-PD-L1 antibodies, transforms transient myocarditis into lethal disease.<sup>8</sup> Depending on the genetic background, PD-1–deficient mice develop dilated cardiomyopathy through the generation of antibodies to Tnl (troponin I)<sup>8,9</sup> or fatal lymphocytic myocarditis with high levels of antimyosin antibodies.<sup>10</sup> Observations in PD-1–deficient mice provided the first clear experimental demonstration of the autoimmune basis of dilated cardiomyopathy in mice.<sup>9</sup> On the basis of these observations, our group developed a cardiac Tnl-induced experimental autoimmune-related myocarditis mouse model that mirrors human disease. Very similar to the phenotype in PD-1–deficient mice, this model features cardiac inflammation and fibrotic scar formation leading to cardiac dysfunction and tissue remodeling.<sup>11</sup>

Specific treatments for autoimmune-related heart disease are rare<sup>12</sup> and because of the broad clinical application of ICIs, there is an urgent need for novel strategies for managing ICI-related myocarditis. Proteasome inhibitors, which inhibit the major proteolytic machinery in all cells, are in consideration for targeting both cancer and autoimmunity.<sup>13–15</sup> The catalytic activity of the proteasome is restricted to its 3  $\beta$ -subunits—namely,  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5—in the standard proteasome, and LMP2 (low-molecular-weight protein 2)/ $\beta$ 1i, MECL1 (multicatalytic endopeptidase complex subunit 1)/ $\beta$ 2i, and LMP7 (low-molecular-weight protein 7)/ $\beta$ 5i in the immune cell resident isoform, the immunoproteasome.<sup>16</sup> Proteasome inhibitors, available for the treatment of multiple myeloma,<sup>17,18</sup> target both the standard proteasome, found in all somatic cells, and the immunoproteasome, found in immune cells (eg, in multiple myeloma cells). More recently, selective inhibitors that specifically block the immunoproteasome emerged as potent compounds to hinder inflammation-driven carcinogenesis.<sup>13</sup> The biological function of the immunoproteasome affects several central aspects of the immune response, such as major histocompatibility complex class I antigen presentation,<sup>19</sup> T-cell differentiation,<sup>20</sup> and cytokine production.<sup>15,21,22</sup> Immunoproteasome proteolysis also controls autoimmune-related inflammation.<sup>15,21</sup> In this study, we investigated how the immunoproteasome

affects TnI-directed autoimmune myocarditis (TnI-AM). We show that selective inhibitors of the immunoproteasome mitigate autoimmune-related myocarditis in mice, and we demonstrate the relevance of autoimmune-related responses for ICI-related myocarditis in 2 patients with cancer.

## METHODS

### Data Availability

All data needed to evaluate the conclusions in the article are present in the article or the [Data Supplement](#). RNA-Seq raw data are available from Dr Meder on request. The R source code for RNA-Seq data analysis is available from Dr Weiner on request. The corresponding authors had full access to all the data in the study and take responsibility for its integrity and the data analysis.

### Patients and Healthy Controls

Written and informed consent was obtained from patients before endomyocardial biopsies were obtained and from healthy donors who agreed to donate blood. The study was performed according to the Declaration of Helsinki. All procedures, as well as blood sampling, were approved by the local ethics committees (EA4/122/14, EA1/189/19, and S-240/2017).

#### Patient 1

A 78-year-old woman with metastatic renal cell carcinoma presented with edema and pain in the upper legs after receiving nivolumab (3 mg/kg, second cycle). The patient was admitted to the hospital because of severe muscle pain 5 days after the second nivolumab treatment. No signs of acute heart failure were documented. The patient was treated with analgesics but not steroids. Postmortem ICI-related myocarditis was demonstrated.<sup>23</sup> Serum was not available from this patient. Postmortem cardiac sections were evaluated microscopically.

#### Patient 2

A 74-year-old man with metastatic non-small-cell lung cancer presented with dyspnea and chest pain after receiving durvalumab for the second time (1.125 mg). Two years previously, tumor therapy included resection of the right upper lung lobe, chemotherapy (carboplatin AUC5 and paclitaxel), and radiation therapy. Durvalumab treatment had been initiated 2 months before admission. ECG showed atrial fibrillation with heart rates of around 110 bpm, an initial manifestation of an intraventricular conduction delay with complete right bundle-branch block and left anterior fascicular block. Elevated levels of TnT (troponin T; 590 pg/mL) and creatine kinase (5119 U/L) were detected. No evidence of pulmonary embolism or pneumonia was found. A coronary angiogram showed stable coronary artery disease and no signs of progression in comparison to an angiogram from 2016. Serial echocardiograms and cardiac magnetic resonance imaging revealed normal left ventricular systolic function, and the magnetic resonance imaging showed no signs of edema. Endomyocardial biopsies obtained from the left ventricle revealed ICI-related myocarditis. After treatment with glucocorticoids (160 mg/d prednisolone), the heart failure symptoms improved.

## Animals and Experimental Autoimmune Myocarditis

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Animal Welfare Act, which is based on the directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes (directive 2010/63/EU). Local state authorities in Karlsruhe and Berlin approved all procedures involving the use and care of animals (German Animal Protection Code: G-161/14, G-0054/18, and G-0103/18). To induce TnI-AM, mice were immunized subcutaneously with a solution of 150 µg murine cardiac TnI peptide HARVDKVDEERYDVEAKVTKNITEADLTQKIYDLRGKFKRPTLRVRIS (Peptide Specialty Laboratories, Heidelberg, Germany) diluted in complete Freund's adjuvant, which was supplemented with 5 mg/mL of *Mycobacterium tuberculosis H37Ra* (Sigma, St Louis, MO).

### Statistics

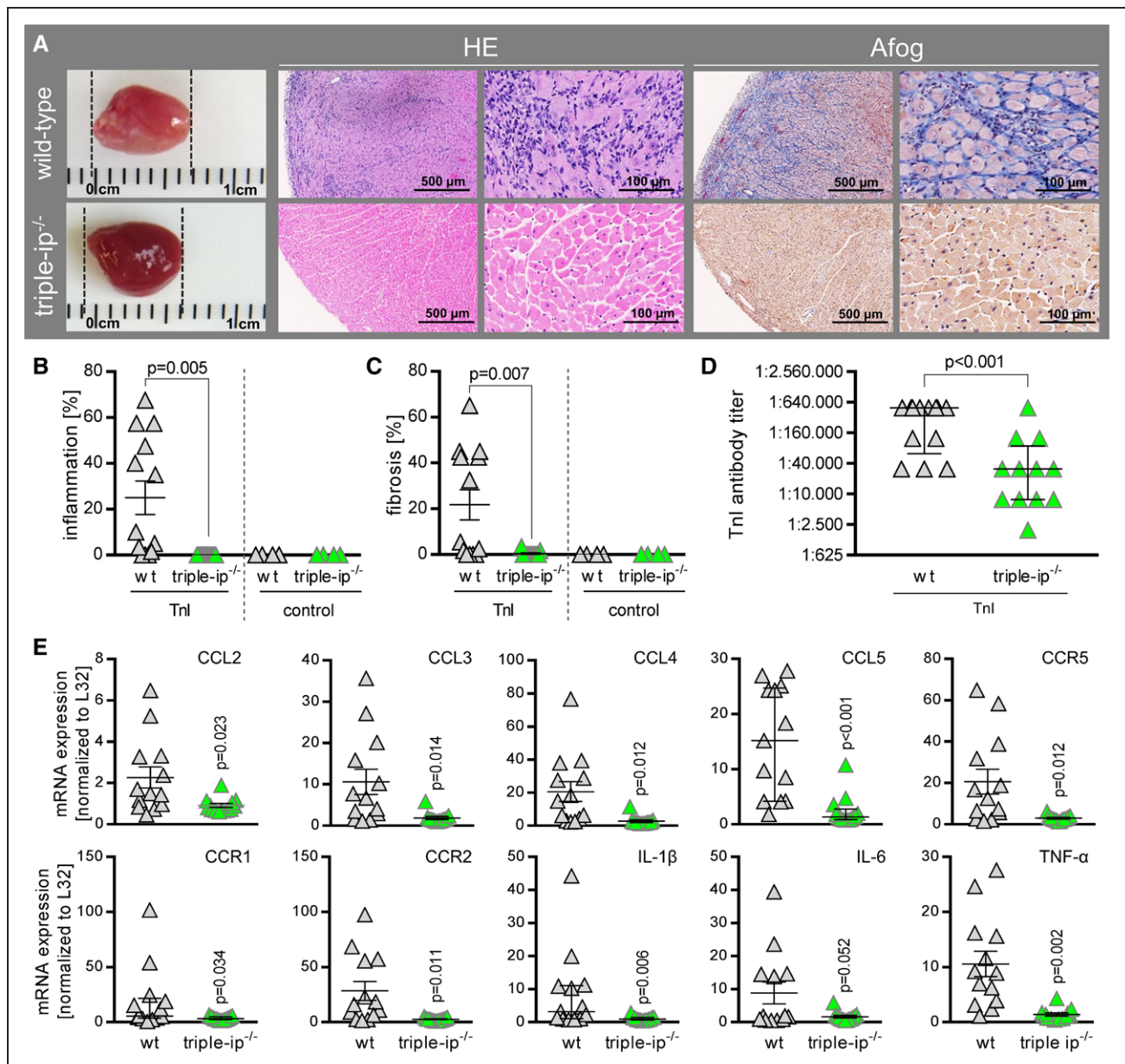
Statistical analysis of the data was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA). All data are plotted as individual points. Normal distribution of the control group was tested using the D'Agostino-Pearson normality test. Data summaries are given as mean±SEM. If data were strongly skewed, they were plotted as median±interquartile range. Paired or unpaired *t* tests were used for 2-group comparisons. If samples had unequal variances (determined by an *F* test), an unpaired *t* test with the Welch correction was used. If the data were skewed, the nonparametric Mann-Whitney test was performed to compare ranks. All tests used were 2-tailed. For multiple group comparison with repeated measurements, 2-way analysis of variance was performed followed by a multiple comparison test. The threshold of significance for all tests was set at 0.05.

## RESULTS

### Absence of the 3 Catalytic Subunits of the Immunoproteasome Abrogates TnI-AM in Mice

For induction of experimental autoimmune myocarditis (TnI-AM) comprising leukocyte recruitment, and fibrotic scar formation (Figure 1A through 1C), mice received several inoculations of an immunogenic cardiac TnI peptide<sup>24</sup> in conjunction with complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis H37Ra*. As a first step toward defining the influence of immunoproteasome-mediated proteolysis on the induction of autoimmune-related myocarditis, TnI-AM was investigated in mice lacking all 3 catalytically active subunits of the immunoproteasome—LMP2, LMP7, and MECL1, called triple-*ip*<sup>−/−</sup>—and in wild-type (wt) controls. TnI-AM was induced to a variable extent in 77% of TnI peptide-immunized wt mice, whereas none of the triple-*ip*<sup>−/−</sup> mice demonstrated significant signs of infiltration (Figure 1A and 1B). Consistently, wt





controls showed collagen deposition, whereas *triple-ip<sup>-/-</sup>* mice had greatly attenuated infiltration and a significant reduction of fibrotic scar formation (Figure 1A and 1C). Control mice that received complete Freund's adjuvant injections but no TnI peptide revealed no histologic or phenotypic signs of TnI-AM, as expected (Figure 1A in the Data Supplement). Corresponding to substantially decreased inflammatory injury of the

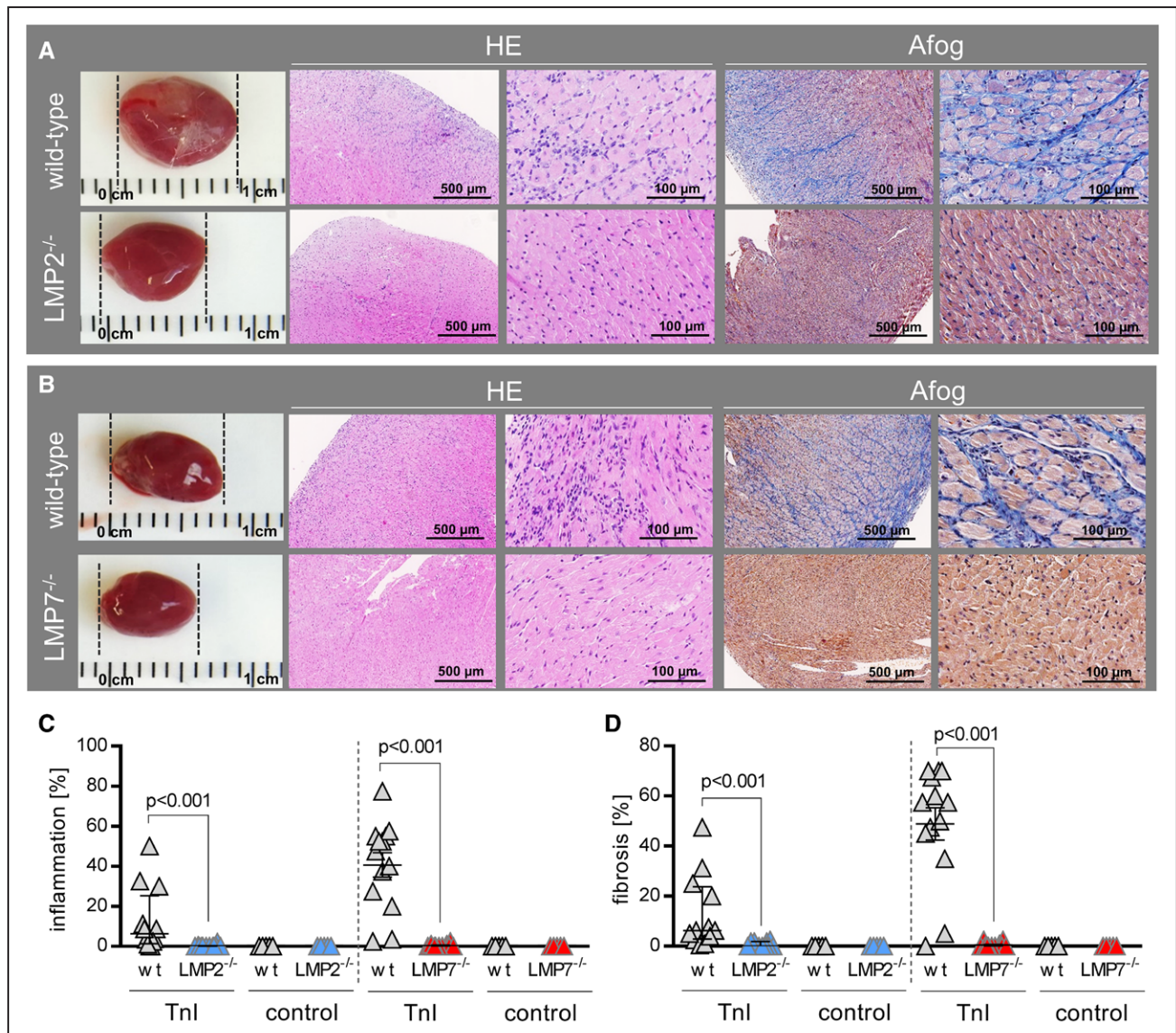
heart tissue, the formation of TnI-directed immunoglobulin G (IgG) antibodies was significantly reduced in *triple-ip<sup>-/-</sup>* mice (Figure 1D).

Members of the chemokine superfamily are crucial for leukocyte recruitment into heart tissue during TnI-AM,<sup>25</sup> and we found elevated levels of expression for the mononuclear cell-attracting molecules CCL2, CCL3, CCL4, and CCL5 in inflamed mouse hearts

(Figure 1E). The expression of the chemokine receptor molecules CCR1, CCR2, and CCR5 was increased consistently. In line with diminished inflammatory damage found in triple-ip<sup>-/-</sup> mice during TnI-AM, hearts from triple-ip<sup>-/-</sup> mice had lower expression of the CC chemokines, as well as their respective receptor molecules, in comparison with wt controls (Figure 1E). In line with this, expression of the proinflammatory cytokines interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  was substantially lower in triple-ip<sup>-/-</sup> mice and found to be within the range of nonpeptide immunized mice (Figure 1E).

## Deletion of LMP2 or LMP7 Mitigates TnI-AM

Triple-ip<sup>-/-</sup> mice experience little to no TnI-AM, so we analyzed single knock outs of LMP2 and LMP7 to determine whether the lack of only 1 of the 3 genes is sufficient to confer the observed protection from TnI-AM. We therefore induced TnI-AM in mice lacking the gene encoding the LMP2 or the LMP7 subunit. As with the triple-ip<sup>-/-</sup> mice, TnI-AM was less severe in both LMP2<sup>-/-</sup> and LMP7<sup>-/-</sup> mice. Ablation of either LMP2 or LMP7 resulted in profound reduction of heart tissue inflammation and fibrosis formation (Figure 2). The effect





of the immunoproteasome on cardiac function during TnI-AM was assessed by echocardiography exemplarily in LMP7<sup>-/-</sup> mice and their littermate controls (Table 1). In comparison with age- and sex-matched naive controls, during TnI-AM, both the stroke volume and the left ventricular ejection fraction were reduced in wt mice. In contrast, LMP7<sup>-/-</sup> mice showed no relevant deterioration of either cardiac function measure during TnI-AM. As another surrogate measure of systolic function, we determined the fractional area change. During TnI-AM, we found a significantly decreased fractional area change in wt mice, whereas LMP7<sup>-/-</sup> mice were protected from this TnI-AM-mediated reduction (Table 1). Together with the low level of inflammation observed, the preservation of stroke volume, left ventricular ejection fraction, and fractional area change after induction of TnI-AM in LMP7<sup>-/-</sup> mice are indicators of a lack of disease-induced cardiac functional deterioration in this strain.

Next, we addressed the question of whether a dysfunctional immunoproteasome in LMP2<sup>-/-</sup> and LMP7<sup>-/-</sup> mice influences the abundance of chemotactic molecules and their respective receptors during TnI-AM. In line with diminished inflammation and fibrosis formation, the expression levels of CCL chemokines were reduced in TnI-immunized LMP2<sup>-/-</sup> and LMP7<sup>-/-</sup> mice compared with wt controls (Figure 3A), and this was accompanied by low levels of CCR1, CCR2, and CCR5 (Figure 3B). The deletion of each single immunoproteasome subunit suppressed the production of proinflammatory cytokines in heart tissue as well. We found a significant decrease of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in both LMP2<sup>-/-</sup> and LMP7<sup>-/-</sup> mouse hearts (Figure 3C). Because autoimmune-related pathology in experimental TnI-AM and in humans involves the activity of CD4<sup>+</sup> T cells,<sup>24,26,27</sup> we also investigated surrogates for CD4<sup>+</sup> T-cell effector function in inflamed heart tissue. TnI-AM resulted in robust upregulation of hallmark cytokines produced by either Th1 cells (interferon- $\gamma$ , IL-2) or Th17 cells (IL-17). Genetic deletion of either LMP2 or

LMP7 reduced the expression of interferon- $\gamma$ , IL-2, and IL-17 in the heart (Figure 3D).

Together with decreased infiltration and lower chemokine and proinflammatory cytokine production in cardiac tissue, the reduction of T-cell cytokines was indicative of diminished effector T-cell responses in mice with a dysfunctional immunoproteasome. Therefore, we investigated whether the immunoproteasome indeed affects the activation status of T cells. Focusing on acute TnI-AM in LMP7<sup>-/-</sup> mice, we analyzed the expression of CD44 and CD62L on the surface of splenic T cells by flow cytometry (Figure 4A and 4B). The abundance of CD44 and of CD62L on CD8<sup>+</sup> T cells was the same in controls and during TnI-AM for both the wt and LMP7<sup>-/-</sup> mice (Figure 4A). The presence of LMP7 had no effect on the levels of the T-cell L-selectin CD62L in naive CD4<sup>+</sup> T cells, but during TnI-AM the expression of CD62L was significantly higher in LMP7<sup>-/-</sup> mice. Cell surface expression of the CD44 antigen, which is a cell surface glycoprotein characteristic of an activated effector or memory T cell, was reduced on CD4<sup>+</sup> T cells in LMP7<sup>-/-</sup> mice, particularly during TnI-AM (Figure 4B). Altogether, these data pointed to lower CD4<sup>+</sup> T-cell activation in LMP7<sup>-/-</sup> mice on TnI immunization. Because checkpoint molecules such as PD-1 regulate the threshold of antigen responses against the heart muscle,<sup>8–10</sup> we investigated whether the immunoproteasome influences PD-1 expression on T cells. We found that splenocytes obtained from LMP7<sup>-/-</sup> mice during TnI-AM had higher PD-1 expression on both CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Figure 4C and 4D). Higher mRNA expression of PD-1 in splenocytes from LMP7<sup>-/-</sup> mice confirmed elevated decoration of CD4<sup>+</sup> T cells with PD-1 molecules (Figure 4E). mRNA expression levels of both CD25 and FoxP3 (forkhead box protein P3), which define the CD4<sup>+</sup> T-cell subset of inducible regulatory T cells (Tregs), were increased in LMP7<sup>-/-</sup> mice during TnI-AM (Figure 4E). Together with unaltered overall CD4 mRNA expression levels during TnI-AM, these data indicate pronounced CD4<sup>+</sup> T-cell differentiation into Tregs in LMP7<sup>-/-</sup> mice.

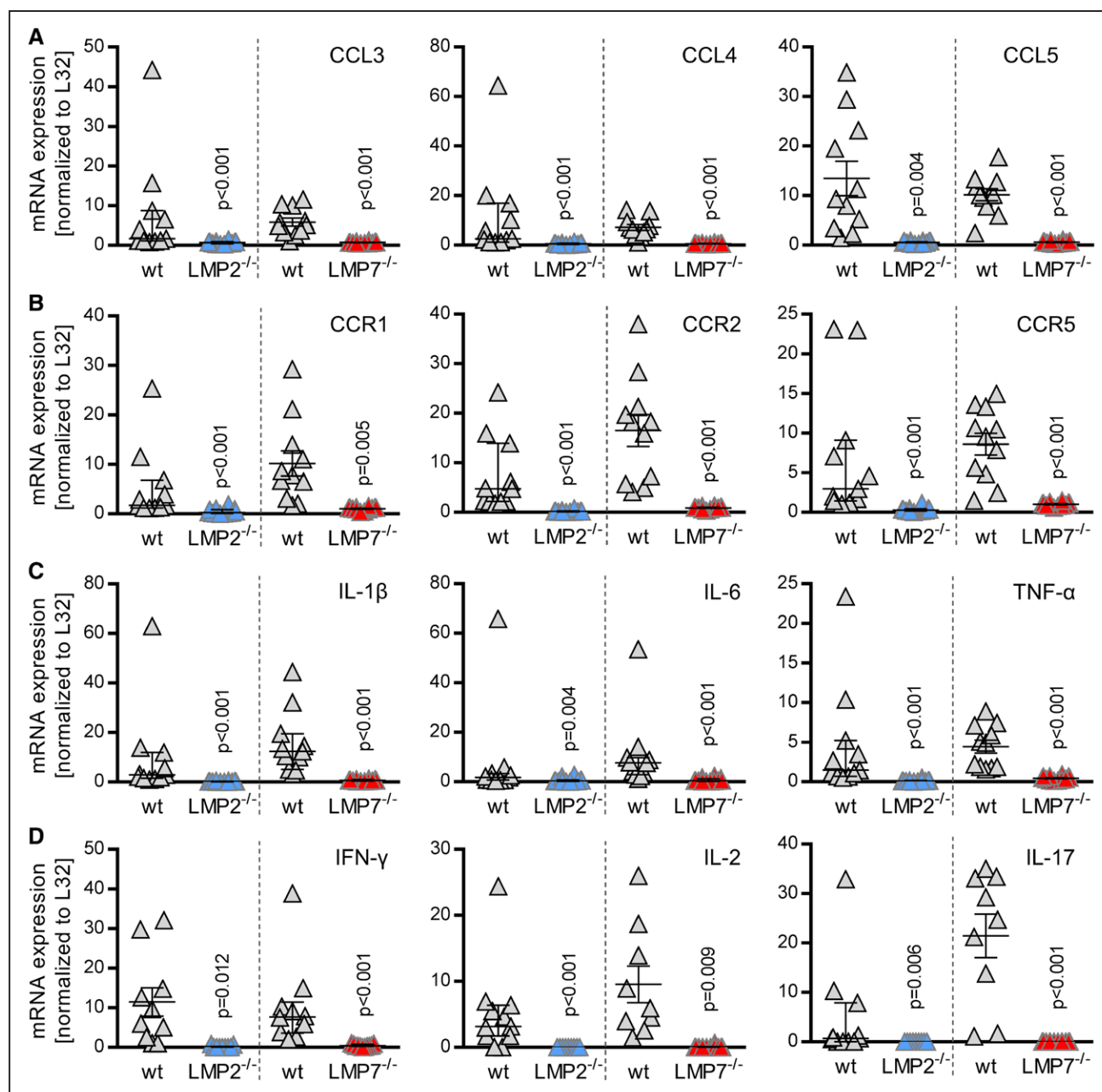
**Table 1. Analysis of Cardiac Function in LMP7<sup>-/-</sup> Mice During Acute TnI-AM**

Parameter	Controls		TnI-AM	
	Wild Type	LMP7 <sup>-/-</sup>	Wild Type	LMP7 <sup>-/-</sup>
Heart rate, bpm	395.1 $\pm$ 10.5	407.7 $\pm$ 9.8	466.9 $\pm$ 7.4*	490.1 $\pm$ 14.2*
Trace left ventricular ejection fraction, %	57.6 $\pm$ 2.5	59.4 $\pm$ 0.7	47.4 $\pm$ 2.1*	52.1 $\pm$ 2.4
Fractional area change, %	49.1 $\pm$ 1.1	49.6 $\pm$ 1.2	36.8 $\pm$ 2.1*†	48.2 $\pm$ 2.6
Stroke volume, $\mu$ L	21.7 $\pm$ 1.2	19.2 $\pm$ 0.8	15.6 $\pm$ 1.4*	18.0 $\pm$ 1.2
Cardiac output, mL/min	8.4 $\pm$ 0.6	7.9 $\pm$ 0.5	7.3 $\pm$ 1.5	8.8 $\pm$ 0.7
Left ventricle internal diameter at diastole, mm	3.5 $\pm$ 0.1	3.4 $\pm$ 0.1	3.29 $\pm$ 0.04	3.25 $\pm$ 0.06
Left ventricle internal diameter at systole, mm	2.4 $\pm$ 0.06	2.3 $\pm$ 0.05	2.4 $\pm$ 0.06	2.4 $\pm$ 0.11

Cardiac function was assessed by echocardiography (Vevo 3100) during the acute state of TnI-AM 28 days after the initial TnI immunization in LMP7<sup>-/-</sup> mice and in wild-type littermate controls (7 in each group). As a control, cardiac function was assessed in age- and sex-matched naive control mice (10 A/J-wild-type and 9 A/J-LMP7<sup>-/-</sup> mice). Female controls were 9 to 10 weeks old, which is equivalent to the age of mice at the acute stage of TnI-AM. Data are mean $\pm$ SEM. Two-way analysis of variance was performed, followed by a Tukey multiple comparison test. AM indicates autoimmune myocarditis; LMP7<sup>-/-</sup>, low-molecular-weight protein 7; and TnI, troponin I.

\*Significant changes between naive controls and TnI-AM mice of the same strain.

†Significant change between wild-type and LMP7<sup>-/-</sup> mice during TnI-AM.



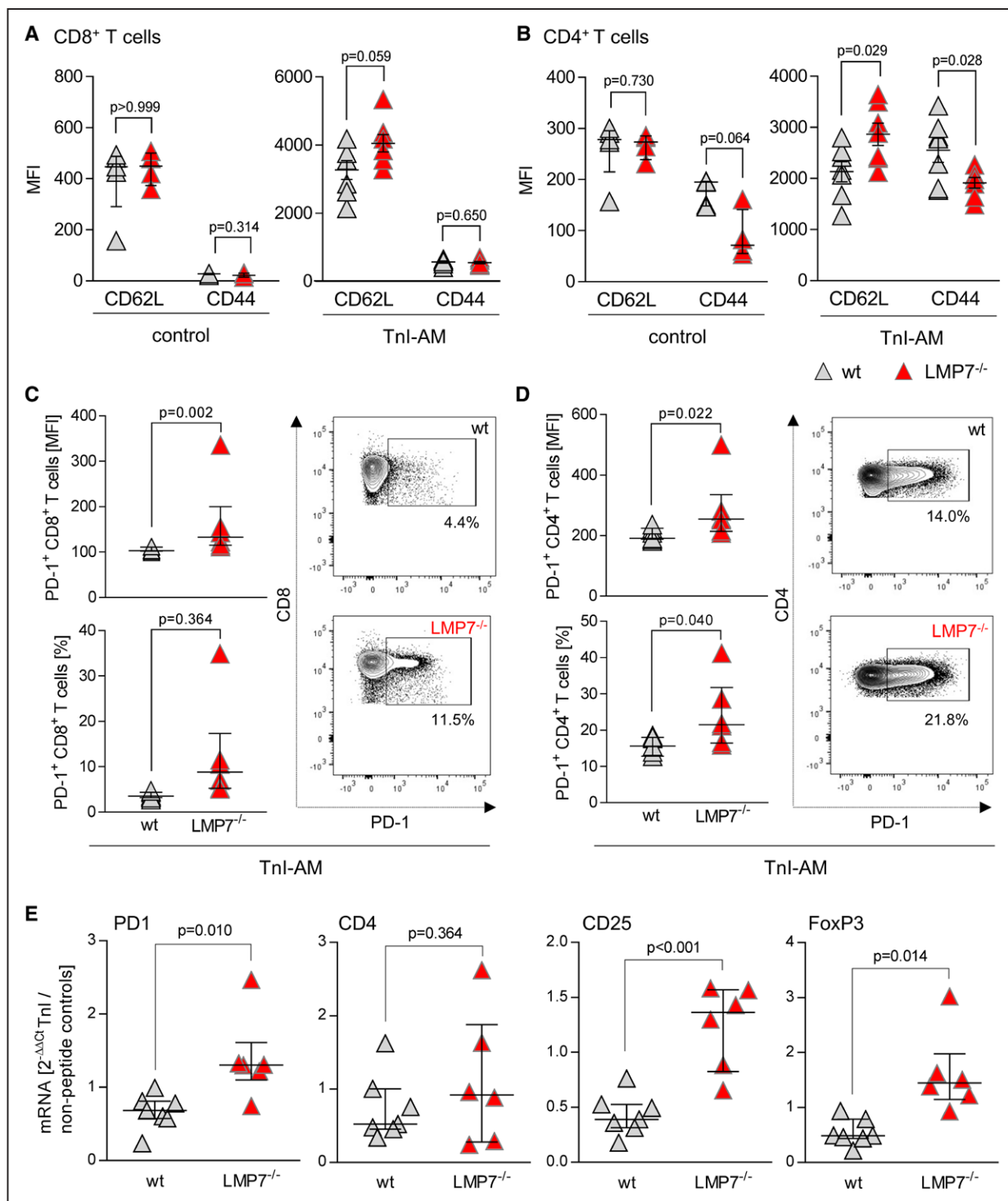
**Figure 3.** Deletion of LMP2 (low-molecular-weight protein 2) or of LMP7 (low-molecular-weight protein 7) suppressed chemokine and cytokine production in the inflamed heart.

Heart tissue obtained from LMP2<sup>-/-</sup> mice, LMP7<sup>-/-</sup> mice, and their respective littermate controls during the acute phase of TnI (troponin I)-directed autoimmune myocarditis (TnI-AM) was homogenized (n=12 for LMP2<sup>+/+</sup>, LMP2<sup>-/-</sup> and LMP7<sup>+/+</sup>; n=13 for LMP7<sup>-/-</sup>). RNA was extracted for quantitative real-time polymerase chain reaction analysis of chemokines (A), chemokine receptors (B), proinflammatory cytokines (C), and hallmark T-cell cytokines (D). The induction of mRNA was normalized to LMP2<sup>+/+</sup> or LMP7<sup>+/+</sup> no-peptide/complete Freund's adjuvant-treated mice (n=5). Normally distributed data (LMP7<sup>-/-</sup> and respective controls: CCL3, CCL4, CCL5, CCR1, CCR2, CCR5, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and interleukin [IL]-17; and LMP2<sup>-/-</sup> and respective controls: CCL5) were plotted as mean±SEM and a t test was performed. Skewed data (LMP2<sup>-/-</sup> and respective controls: CCL3, CCL4, CCR1, CCR2, CCR5, IL-1β, IL-6, TNF-α, IFN-γ, IL-2, and IL-17; and LMP7<sup>-/-</sup> and respective controls: IL-1β, IL-2, and IL-6) was plotted as median±interquartile range and a Mann-Whitney test was performed. P values are indicated in each graph. wt indicates wild-type.

## Blockade of the Immunoproteasome by ONX 0914 Diminishes TnI-AM

Because deletion of either LMP7 or LMP2 was sufficient to prevent disease development during TnI-AM (Figures 2 and 3), we investigated whether ONX 0914, a potent third-generation inhibitor selective for the

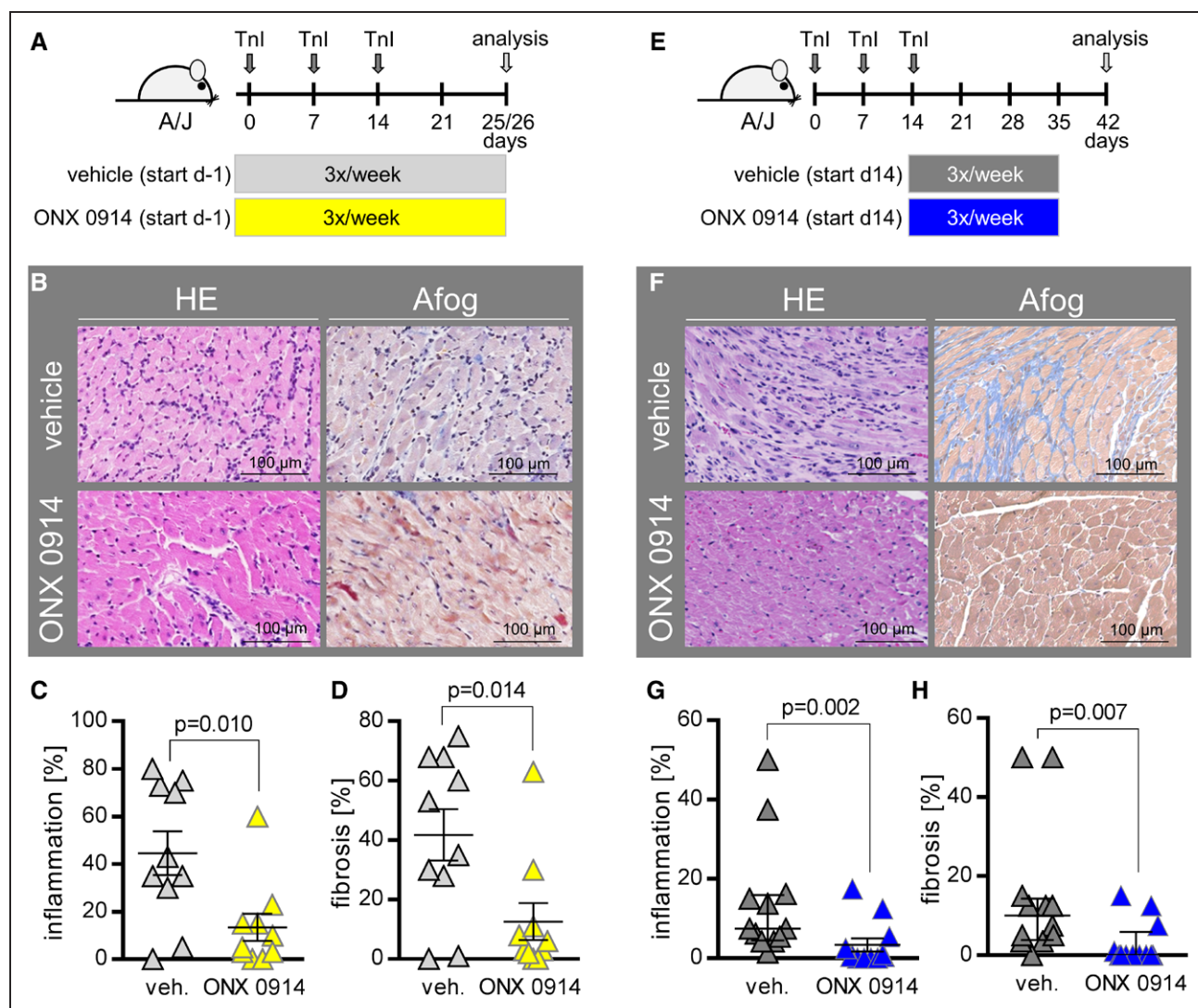
immunoproteasome,<sup>15</sup> influences TnI-AM. The mice received ONX 0914 a total of 3 times per week for 4 weeks, starting 1 day before TnI immunization (Figure 5A). As indicated by an upward shift of the respective protein band in Western blot analysis of splenic homogenates, ONX 0914 blocked LMP7 nearly completely and LMP2 partially (Figure IIIA in the Data Supplement). In



**Figure 4.** Deletion of LMP7 (low-molecular-weight protein 7) shaped CD4<sup>+</sup> T-cell responses during TnI (troponin I)-directed autoimmune myocarditis (TnI-AM).

LMP7<sup>-/-</sup> mice and their respective controls were immunized according to the protocol described and killed after 28 days (7 wild-type [wt]; 6 LMP7<sup>-/-</sup> mice) for isolation of splenocytes. In a separate experiment, splenocytes were isolated from sex-matched naive LMP7<sup>-/-</sup> mice and wt controls (5 per group). T cells were defined as CD3<sup>+</sup> and further distinguished as CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup>. Cellular surface expression of CD44, CD62-L (A and B), and PD-1 (programmed cell death protein 1; C and D) was determined by flow cytometry. In addition to median fluorescence intensity (MFI), the relative frequency of PD-1-expressing T cells was determined, and representative contour plots are depicted showing the abundance of PD-1-expressing CD8<sup>+</sup> and CD4<sup>+</sup> T cells in wt and LMP7<sup>-/-</sup> mice during TnI-AM. PD-1 expression was not affected in naive LMP7<sup>-/-</sup> mice in comparison to naive wt controls (data not shown). Normally distributed data (CD44 on CD8<sup>+</sup> T cells: naive and TnI-AM; CD44 and CD62L on CD4<sup>+</sup> T cells: TnI-AM; percent PD-1<sup>+</sup> CD8<sup>+</sup> T cells: TnI-AM) were plotted as mean±SEM and a t test was performed. Skewed data (CD62L on naive CD8<sup>+</sup> and CD4<sup>+</sup> T cells; CD44 on naive CD4<sup>+</sup> T cells; percent PD-1<sup>+</sup> CD8<sup>+</sup> T cells: TnI-AM; MFI PD-1 on CD8<sup>+</sup> and CD4<sup>+</sup> T cells: TnI-AM) was plotted as median±interquartile range and a Mann-Whitney test was performed. P values are indicated in each graph. E, mRNA expression levels of the indicated target genes (PD-1, CD4, CD25, FoxP3 [forkhead box protein P3]) were determined in splenic tissue during TnI-AM and normalized to respective nonpeptide, complete Freund's adjuvant-treated, age- and sex-matched controls (2 wt mice; 2 LMP7<sup>-/-</sup> mice) using the 2<sup>-ΔΔCt</sup> method. Data are plotted as mean±SEM and a t test was performed (for FoxP3, a Welch correction was performed).





**Figure 5. Inhibition of the immunoproteasome by ONX 0914 diminished TnI (troponin I)-directed autoimmune myocarditis (TnI-AM).**

**A**, Wild-type (wt) A/J mice ( $n=20$ ) were divided into 2 groups that received either vehicle or ONX 0914 a total of 3 times a week starting 1 day before the first TnI immunization ( $n=10$  for vehicle and  $n=10$  for ONX 0914). On days 7 and 14, mice received a second and third immunization, respectively. Mice were killed 25 and 26 days after the first immunization (equal distribution of both treatment groups). **B**, On induction of TnI-AM, hearts were removed. Representative micrographs of hematoxylin-eosin (HE)-stained or acid fuchsin orange G (Afog)-stained heart tissue sections are demonstrated. Heart tissue slides were scored microscopically for inflammation (**C**) and fibrosis (**D**) as described. Data summary is plotted as mean  $\pm$  SEM and a  $t$  test with Welch correction was performed.  $P$  values are indicated in each graph. **E**, Wild-type A/J mice ( $n=24$ ) were immunized with TnI peptide and immunization was repeated after 7 and 14 days. Once autoimmune injury of the heart was evident, all immunized mice were divided into 2 groups that received either vehicle or ONX 0914 a total of 3 times a week starting on day 14 (equal distribution of both treatment groups). Mice were killed on day 42. **F**, Representative micrographs of HE-stained or Afog-stained heart tissue sections are demonstrated. Heart tissue slides were microscopically scored for inflammation (**G**) and fibrosis (**H**) as described. Data summary is plotted as median  $\pm$  interquartile range and a Mann-Whitney test was performed.  $P$  values are indicated in each graph.

line with our findings in all immunoproteasome-deficient mouse strains, analysis of heart tissue during TnI-AM revealed distinct differences between ONX 0914-treated and vehicle-treated mice. Histologic staining of heart tissue (Figure 5B) and subsequent quantitative scoring of the inflamed area as well as of the collagen content (Figure 5C and 5D) demonstrated myocardial injury in vehicle-treated A/J mice; in contrast, only moderate infiltration and fibrosis formation were observed after inhibitor treatment. To obtain more information on how ONX 0914 mitigates TnI-AM, infiltrating immune cells of hearts from vehicle-treated and ONX 0914-treated mice

were analyzed quantitatively by flow cytometry. Vehicle-treated A/J mice demonstrated a high abundance of CD45<sup>+</sup>/CD11b<sup>high</sup> myeloid cells on TnI immunization and only a minor infiltration of lymphoid cells. ONX 0914 reduced this infiltration of CD45<sup>+</sup>/CD11b<sup>high</sup> myeloid cells significantly. Further differentiation of these myeloid cells revealed that ONX 0914 inhibited infiltration of Ly6C<sup>high</sup> inflammatory monocytes significantly. The abundance of Ly6C<sup>low</sup> monocytes and macrophages was lower under the influence of ONX 0914 (Figure III B and III C in the Data Supplement, with the gating strategy for flow cytometry of cardiac inflammatory cells being presented in

Figure II in the Data Supplement). We also tested whether ONX 0914 affects cardiac disease once immune cells have already infiltrated the heart. Therefore, TnI-AM was induced in A/J mice and ONX 0914 treatment was postponed until after the third immunization on day 14. The noncontrol mice received ONX 0914 a total of 3 times a week and mice were killed after 42 days (Figure 5E). Histologic investigation of mouse hearts demonstrated that ONX 0914 had a beneficial effect on the level of inflammation, even after TnI-triggered autoimmune injury of the heart had already taken place. After 42 days, we detected mitigation of perimyocardial tissue injury in ONX 0914-treated mice, as indicated by low-grade inflammation and reduced collagen deposition (Figure 5F through 5H). To investigate the influence of ONX 0914 treatment, initiated on day 14, on cardiac function, we performed echocardiography in the same groups on days 28 and 42. At the acute stage of TnI myocarditis on day 28, ONX 0914 treatment improved cardiac function, as reflected by higher stroke volume. Further supportive evidence of functional benefit achieved in the ONX 0914 group came from improvement of the fractional area change and the peak mitral annular systolic and early diastolic velocity, the latter resulting in a lower ratio between early mitral inflow velocity and mitral annular early diastolic velocity (E/e'). Left ventricular ejection fraction showed a similar but insignificant improvement in the ONX 0914 group. At the advanced stage on day 42, in comparison with day 28, cardiac infiltration and fibrosis formation was less severe and echocardiographic parameters were

similar in vehicle-treated and ONX 0914-treated mice (Table 2). The naive control groups presented in Table 1 and in Table I in the Data Supplement had a stroke volume similar to that of mice at an advanced state of TnI-AM (Table 2).

## ONX 0914 Reduces TLR (Toll-Like Receptor)–Triggered Cytokine Production in Human Monocytes

Monocytes produce inflammatory and chemotactic cytokines, and, in myocarditis, secreted molecules such as IL-6 activate expansion of self-reactive CD4<sup>+</sup> T cells and their differentiation into Th17 effector cells. These cells, in turn, have detrimental effects on autoimmune-related myocarditis.<sup>27–29</sup> Moreover, monocytes are the main producers of CCL chemokines required for the development of TnI-AM.<sup>25</sup> Therefore, we investigated whether immunoproteasome inhibitors influence cytokine production by monocytes, as suggested by our results. Human CD14<sup>+</sup> blood monocytes isolated from healthy donors showed a robust induction of CCL3, CCL4, CXCL2, IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 production when activated with synthetic ligands of TLR2, TLR7/8, and TLR4, which are involved in monocyte activation, leading to heart-specific autoimmunity.<sup>27,30</sup> Blockade of the immunoproteasome by ONX 0914 greatly inhibited the transcriptional activity of these chemokines/cytokines, with the most effective suppression of cytokine expression being observed for TLR2 (Figure 6).

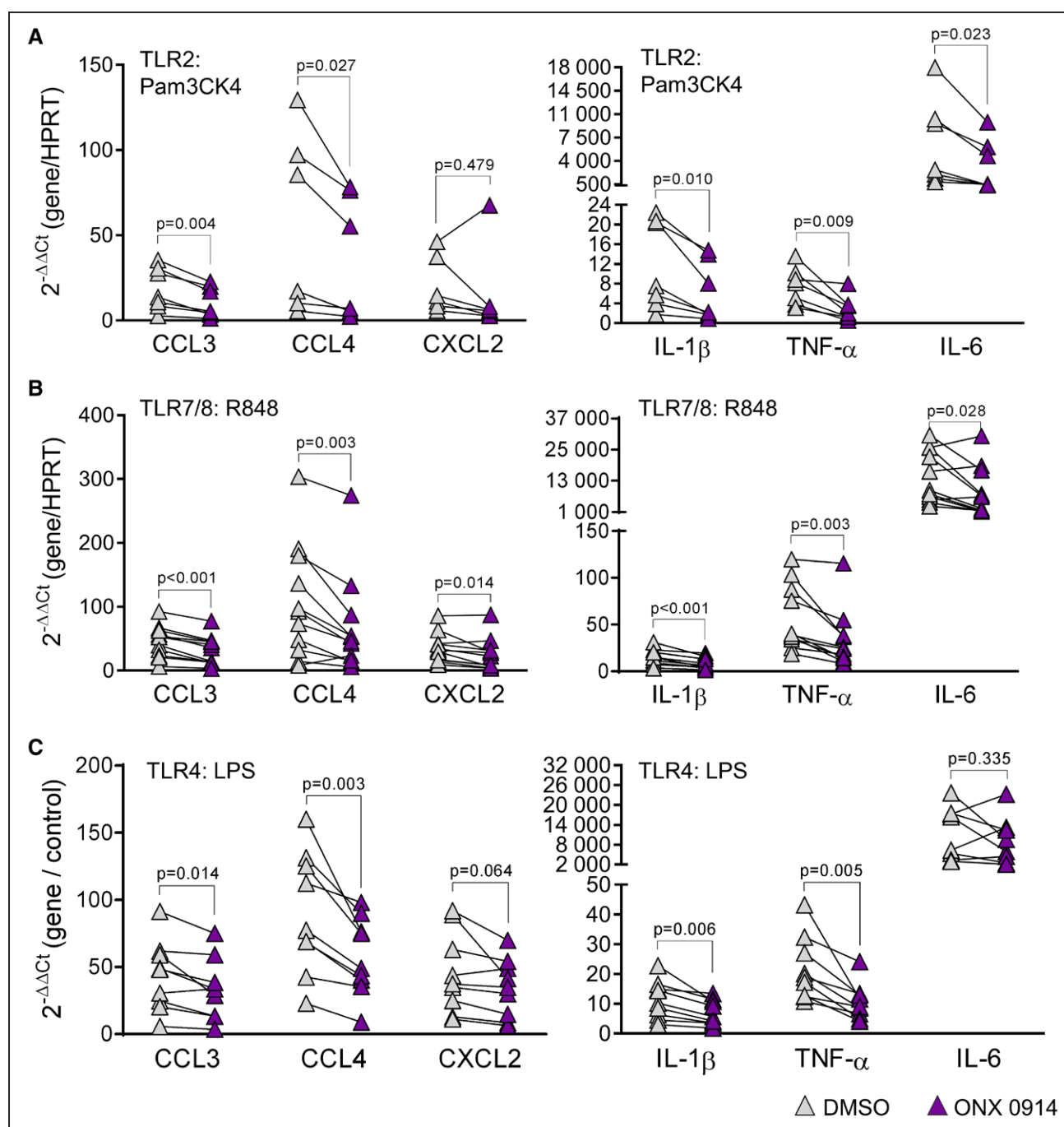
**Table 2.** Effect of ONX 0914 on Cardiac Function During TnI-AM

Parameter	Day 28		Day 42	
	Vehicle	ONX 0914	Vehicle	ONX 0914
Heart rate, bpm	429 $\pm$ 13	401 $\pm$ 20	418 $\pm$ 16	452 $\pm$ 18
Trace left ventricular ejection fraction, %	59.7 $\pm$ 2.9	66.5 $\pm$ 3.4	67.1 $\pm$ 1.5	66.7 $\pm$ 3.0
Fractional area change, %	51.0 $\pm$ 3.1	63.4 $\pm$ 3.4*	49.7 $\pm$ 2.6	55.3 $\pm$ 2.3
MV s', mm/s	17.3 $\pm$ 0.9	23.0 $\pm$ 1.9*	25.7 $\pm$ 2.0	25.4 $\pm$ 1.3
Stroke volume, $\mu$ L	14.2 $\pm$ 2.3	21.0 $\pm$ 2.0*	20.8 $\pm$ 1.3	18.5 $\pm$ 1.7
Left ventricle internal diameter at diastole, mm	2.8 $\pm$ 0.1	3.2 $\pm$ 0.2	3.2 $\pm$ 0.1	3.3 $\pm$ 0.1
Left ventricle internal diameter at systole, mm	2.0 $\pm$ 0.1	2.1 $\pm$ 0.1	2.1 $\pm$ 0.1	2.1 $\pm$ 0.1
MV E, mm/s†	695.3 $\pm$ 33.1	728.0 $\pm$ 81.2	744.0 $\pm$ 50.7	651.8 $\pm$ 36.8
MV e', mm/s†	14.1 $\pm$ 1.3	22.6 $\pm$ 2.0*	30.3 $\pm$ 3.3	32.4 $\pm$ 2.7
MV E/e'†	51.0 $\pm$ 3.7	33.1 $\pm$ 1.9†	23.6 $\pm$ 1.3	21.6 $\pm$ 3.5
MV ejection time, ms†	55.5 $\pm$ 2.0	51.8 $\pm$ 1.5	44.7 $\pm$ 1.8	46.6 $\pm$ 1.1
MV decel', ms†	20.7 $\pm$ 2.5	25.5 $\pm$ 1.5	18.8 $\pm$ 1.0	17.0 $\pm$ 1.2
Isovolumic relaxation time, ms†	18.8 $\pm$ 0.9	17.9 $\pm$ 1.5	14.2 $\pm$ 0.6	15.7 $\pm$ 0.6

Mice were treated with ONX 0914 starting 14 days after the initial TnI peptide immunization and underwent echocardiography (Vevo 3100) to monitor left ventricular systolic and diastolic function on days 28 and 42 (all mice immunized with TnI peptide on day 0, 7, and 14 had elevated serum TnT levels on day 28: 7 vehicle, 7 ONX 0914). Data are mean $\pm$ SEM. Unpaired *t* tests were conducted and ONX 0914 treatment had no significant effect on cardiac function (Table I in the Data Supplement). Peak mitral valve (MV) systole (s') and early diastole (e') velocity were determined by tissue Doppler imaging. Isovolumic relaxation time, MV ejection time, MV deceleration time (MV decel'), and early MV inflow (E) velocity were determined by pulse-wave Doppler at MV. AM indicates autoimmune myocarditis; E/e', ratio between early mitral inflow velocity and mitral annular early diastolic velocity; TnI, troponin I; and TnT, troponin T.

\*Significant changes between vehicle-treated and ONX 0914-treated mice on day 28.

†Measure to assess diastolic function of the left ventricle.



**Figure 6. ONX 0914 reduced TLR (Toll-like receptor)-induced cytokine production in human monocytes.**

Monocytes from 7 to 11 healthy donors were treated with ONX 0914 or dimethyl sulfoxide (DMSO) before stimulation with (A) TLR2 agonist Pam3CK4 (a synthetic triacylated lipopeptide), (B) the TLR7 agonist resiquimod R848 (an imidazoquinoline), or (C) the TLR4 agonist lipopolysaccharide (LPS). After 4 hours, chemotactic (left diagram) and proinflammatory cytokine expression (right diagram) was determined for the indicated genes by TaqMan quantitative polymerase chain reaction. Data points represent the mRNA expression of the respective gene for each donor in TLR2-, TLR7/8-, and TLR4-activated cells. The expression of mRNA was normalized to the mRNA level in cells that were not treated with the respective TLR agonist for both DMSO-treated and ONX-0914-treated cells, respectively ( $2^{-\Delta\Delta C_t}$  method). Paired *t* tests were performed on  $2^{-\Delta\Delta C_t}$  values between TLR-activated DMSO-treated and ONX-0914-treated cells and *P* values are depicted. HPRT indicates hypoxanthine-guanine phosphoribosyltransferase; IL, interleukin; and TNF, tumor necrosis factor.

## Evidence for Heart-Specific Autoimmune Reactions in Cases of ICI-Related Myocarditis

To investigate the overall activation of heart-directed autoimmunity in ICI-related myocarditis, heart tissue from

a patient with PD-1 inhibitor-related myocarditis (patient 1) and a patient with PD-1L inhibitor-related myocarditis (patient 2) were investigated by immunohistochemistry. Patchy lesions with lymphocytic (CD3) and monocytic/macrophage (CD68) infiltration showed greatly increased expression of both LMP2 and LMP7 subunits, indicating



high immunoproteasome activity in ICI-related myocarditis (Figure 7A through 7C), similar to TnI myocarditis in mice (Figure IV in the Data Supplement). We examined these 2 cases of ICI-related myocarditis for signs of heart-specific autoimmune reactions. Immunohistochemical analysis revealed diffuse deposition of IgG in inflamed foci (Figure 7A and 7B), whereas nonaffected heart tissue was negative for IgG. To confirm the presence of an autoimmune reaction against the heart, we examined the Th17 immunophenotype in ICI-related myocarditis, and found IL-17<sup>+</sup> cells in the heart (Figure 7A and 7B). Gene set enrichment analysis of RNA-Seq data, which we obtained from endomyocardial biopsies of patient 2, in comparison with 2 control samples, revealed a significant upregulation of specific inflammatory modules such as the major histocompatibility complex–TLR7–TLR8 cluster or of genes involved in antigen presentation, cell adhesion, and B- and T-cell activation (Figure 7C and Figure V in the Data Supplement). To compare the inflammatory gene signature of this ICI-related myocarditis case with autoimmune myocarditis in mice, on the basis of the inflammatory signature that we detected in TnI-AM, we defined a gene set comprising markers for immunoproteasome expression, TLR-mediated activation of monocytes, chemokine and cytokine responses, as well as T- and B-cell activation (Table II in the Data Supplement). We found a significant enrichment of this autoimmunity gene set in endomyocardial biopsies from patient 2 (Figure 7C and 7D), suggesting that similar inflammatory pathways are involved in both ICI-related myocarditis and experimental TnI-AM in mice. More information on the autoimmune phenotype in ICI-related myocarditis was obtained from analysis of the humoral immune response in patient 2. Serum exhibited high-titer IgG activity of 1:160 against human TnI, as demonstrated by enzyme-linked immunosorbent assay and confirmed by Western blotting (lane 2, Figure 7E). We also investigated whether this TnI-directed humoral immune response in ICI-related myocarditis is specific for the immunogenic epitope that is known to induce autoimmune-mediated infiltration of immune cells to the heart in the mouse model of TnI-AM.<sup>24</sup> We performed Western blotting with serum from patient 2 of a 50mer TnI peptide harboring the myocarditogenic TnI epitope VDKVDEERYDVEAKVTKN and found specific detection of this immunogenic peptide (lane 1, Figure 7E). This finding defines TnI as an autoantigen in this case of ICI-related myocarditis and supports the applicability of the TnI-AM mouse model to investigate autoimmune-related myocarditis in ICI therapy. Altogether, our data show that, in these 2 patients, heart-specific autoimmune reactions are active in ICI-related myocarditis.

## DISCUSSION

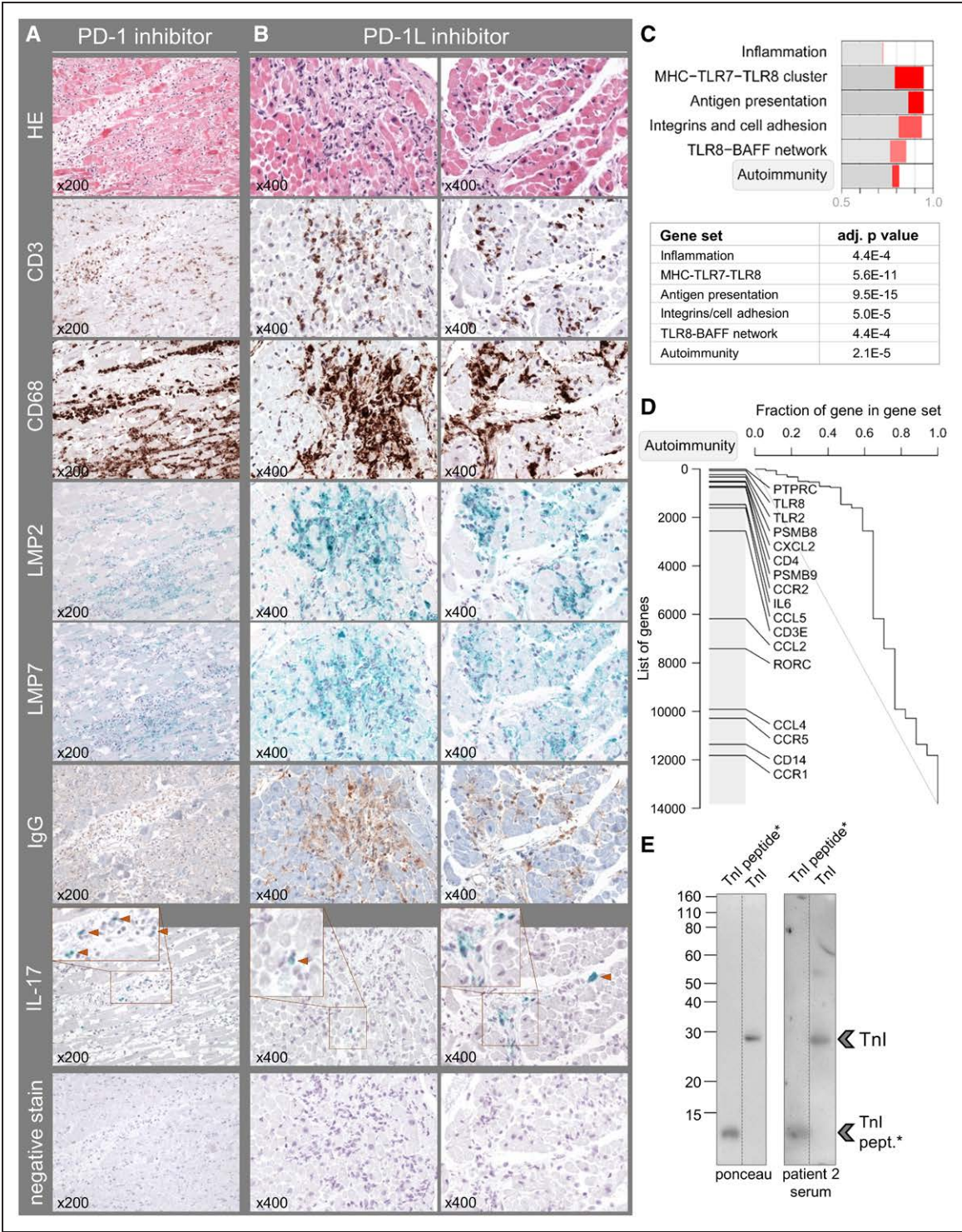
Advances in cancer immunotherapy using ICI to treat metastatic disease have improved survival tremendously.

Blockade of central immune checkpoints such as the PD-1:PD-1L pathway unleashes tumor-specific T cells but also attenuates signals regulating T-cell tolerance, leading to the activation of self-reactive T-cell effector function and triggering injury of heart tissue.<sup>1,2,8–10</sup> Autoimmune-related myocarditis has emerged as a high-risk adverse event in ICI therapy.<sup>5,7</sup> In this study, we focused on the pathophysiologic functions of the immunoproteasome in an experimental model of TnI-AM. As summarized in Figure 8, we found that the immunoproteasome stimulates the activation and expansion of self-reactive CD4<sup>+</sup> T cells and suppresses inhibitory signals. In monocytes, the protease regulates TLR signaling, leading to high expression of proinflammatory cytokines, thereby steering CD4<sup>+</sup> T-cell differentiation toward Th17 and Th1 effector cells. These effectors reduce the Treg pool and stimulate autoantibody production. These processes ultimately result in severe inflammatory heart tissue damage, fibrotic scar formation, and cardiac dysfunction. Elimination of immunoproteasome–protease activity by ONX 0914 elevates the threshold of cardiac autoantigen responses, recalibrating the balance of the immune system and avoiding overactivation, thereby reducing cardiac injury and maintaining function (Figure 8).

## Regulation of Autoimmune-Related T-Cell Effector Function by the Immunoproteasome

CD4<sup>+</sup> T cells are the main trigger of autoimmune-related myocarditis.<sup>24,33</sup> Activation of self-reactive CD4<sup>+</sup> T cells targeting cardiac proteins such as TnI<sup>24</sup> or myosin<sup>33</sup> requires antigen presentation by dendritic cells (DCs), and occurs only when DCs are stimulated through TLR-mediated signaling.<sup>33</sup> Self-antigens released from cardiomyocyte-derived proteins on tissue damage are sensed by DCs through TLRs, and the resulting phenotypic and functional changes in DCs trigger their migration and facilitate antigen presentation. The immunoproteasome is a necessary component of the signaling pathways between the TLRs and MyD88 (myeloid differentiation primary response 88), which integrates their signals.<sup>15,34</sup> Elimination of immunoproteasome activity compromises TLR–MyD88 pathways,<sup>35</sup> resulting in impaired antigen presentation, thereby limiting autoimmune-related T-cell effector activity and attenuating cardiac injury, as reported in this study in mice with immunoproteasome deficiency or blocked immunoproteasome activity.

Beyond antigen presentation by DCs, immunoproteasome-dependent biological processes involve CD4<sup>+</sup> T-cell activation, proliferation,<sup>36</sup> and exhaustion.<sup>35</sup> LMP7<sup>−/−</sup> mice undergoing TnI-AM have higher CD62L and lower CD44 expression on CD4<sup>+</sup> T cells than wt control mice, which indicates a lower state



**Figure 7. Immunoproteasome expression, immunoglobulin G (IgG) deposits, and Th17 cells indicated heart-directed autoimmune activity in cases of immune checkpoint inhibitor-related myocarditis.**

**A**, Postmortem diagnosis of a fatal case of nivolumab-related myocarditis (patient 1).<sup>23</sup> **B**, Endomyocardial biopsies from durvalumab-related myocarditis (patient 2). For patient 2, 2 different foci from the same biopsy are depicted. Staining with hematoxylin-eosin (HE) shows acute lymphocytic infiltration. The inflammatory infiltrate included CD3-positive T cells and CD68-positive macrophages. Myocardial tissue stained for the immunoproteasome subunits LMP2 (low-molecular-weight protein 2) and LMP7 (low-molecular-weight protein 7) indicated high immunoproteasome expression. Heart sections stained with antihuman IgG illustrate deposition of IgG. The blue arrows in sections stained with antibody against interleukin (IL)-17 point toward Th17 cell infiltration. **C**, Gene set enrichment analysis results of RNA-Seq data from endomyocardial biopsies of patient 2 (for information regarding overall differential gene expression, refer to [Table III in the Data Supplement](#)). Each bar corresponds to a single gene module from the tmod package.<sup>31</sup> The gene set “autoimmunity” was operator-defined based on the inflammatory signature detected in the mouse model of TnI (troponin I)-directed autoimmune myocarditis (TnI-AM; [Table II in the Data Supplement](#)). The length of the bar represents effect size (enrichment strength as area under the curve [AUC]). The color intensity corresponds to P value; adjusted P values are shown below the color-coded image plot. With the exception of the inflammation module, all selected modules have a P<0.01 and AUC>0.75. (Continued)



of T-cell activation in LMP7<sup>-/-</sup> mice, similar to findings for CD8<sup>+</sup> T cells during lymphocytic choriomeningitis virus infection.<sup>37</sup> PD-1 delivers inhibitory signals specifically to CD4<sup>+</sup> T cells that regulate T-cell tolerance and protects from autoimmune-related myocarditis.<sup>38</sup> Therefore, increased PD-1 levels, as reported here for LMP7<sup>-/-</sup> CD4<sup>+</sup> T cells during Tnl-AM, corroborate the pathophysiologic relevance of low T-cell effector activity as the trigger of mitigated pathology in immunoproteasome-deficient mice. In both mice and patients with autoimmune-related myocarditis and dilated cardiomyopathy, specific CD4<sup>+</sup> T-cell subsets, in particular Th17 cells, contribute to cardiac remodeling processes.<sup>27,29</sup> In 2 cases of ICI-related myocarditis studied by us, we found IL-17-producing T cells in the heart as well, providing evidence for heart-specific immune responses in these cases of ICI cancer immunotherapy. The immunoproteasome pushes CD4<sup>+</sup> T-cell differentiation toward higher Th17 and Th1 expansion.<sup>20</sup> In immunoproteasome-deficient strains, we demonstrate reduced Th17 (IL-17) and Th1 (interferon- $\gamma$ ) hallmark cytokines during Tnl-AM, indicating diminished self-reactive CD4<sup>+</sup> T-cell effector function on elimination of the immunoproteasome.

Monocytes are the main producers of proinflammatory cytokines, which govern expansion of autoreactive CD4<sup>+</sup> T cells and their differentiation into Th17 cells.<sup>27</sup> Induction of Th17 cells can also occur when memory CD4<sup>+</sup> T cells encounter TLR-activated monocytes.<sup>39</sup> Immunoproteasome inhibitors, however, efficiently block the production of proinflammatory cytokines, particularly in TLR2- and TLR7/8-activated monocytes. This finding is important because both TLR2- and TLR7/8-activated monocytes can trigger a Th17 immunophenotype in patients with autoimmune myocarditis.<sup>27,30</sup> Similar to human myocarditis or dilated cardiomyopathy,<sup>27</sup> in the presence of elevated proinflammatory cytokines, decreased Tregs characterize the Th17 immunophenotype in autoimmune Tnl-related myocarditis. In immunoproteasome-deficient mice, however, lower IL-6 and IL-1 $\beta$  production reshapes CD4<sup>+</sup> T-cell differentiation toward elevated Treg abundance during Tnl-AM. CD4<sup>+</sup> Tregs mitigate autoimmune-related cardiac disease.<sup>27</sup> In line with high PD-1 expression and elevated Treg abundance in LMP7<sup>-/-</sup> mice during Tnl-AM, PD-1 and

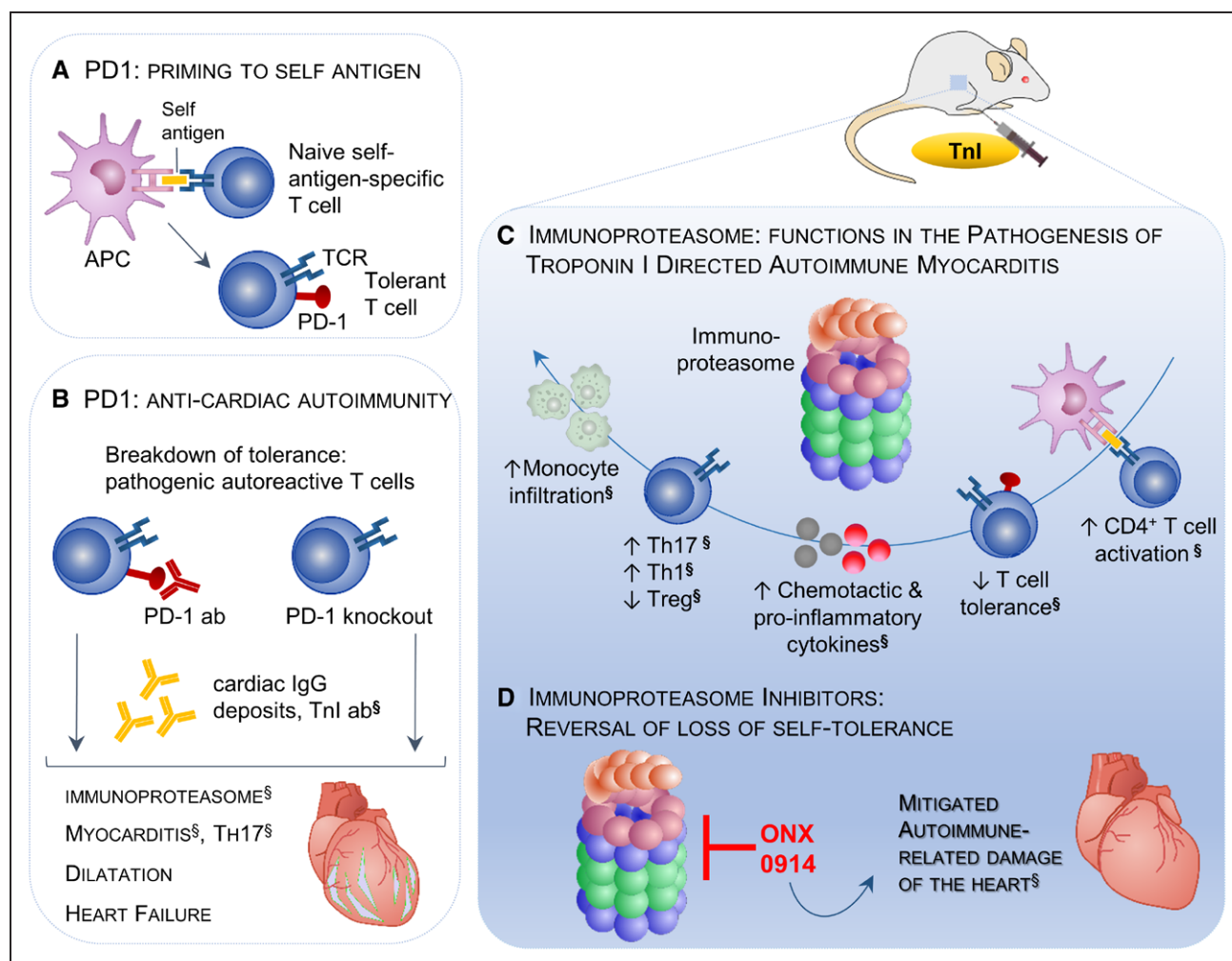
its ligands also promote the development and function of Tregs, and thereby protect against potentially pathogenic self-reactive effector T cells.<sup>1</sup> Altogether, reduced T-cell activation, elevated T-cell tolerance, and limitation of Th17 immunophenotype are beneficial aspects of reducing immunoproteasome function in autoimmune-related myocarditis. It remains uncertain to what extent protection from Tnl-AM in immunoproteasome-deficient or ONX 0914-treated mice can be attributed to an altered effector CD4<sup>+</sup> T-cell repertoire. A number of different mechanisms have been described to explain the function of the immunoproteasome in effector CD4<sup>+</sup> T cells in the context of different diseases.<sup>14,20</sup> What these mechanisms have in common is that impaired immunoproteasome function blocks autoimmune tissue damage.<sup>15,21</sup>

### Function of the Immunoproteasome in Innate Myeloid Cells and Effect on Myocarditis

CD11b<sup>+</sup> monocytes/macrophages are the major heart-infiltrating immune cells during Tnl-AM and are central for mediating tissue damage and fibrotic scarring.<sup>40</sup> Results from experimental and clinical trials indicate that chemokines are crucial for the pathogenesis leading to heart failure. By binding to their receptors CCR2 and CCR5, CCL2 and CCL3 stimulate inflammatory heart tissue injury in Tnl-AM, and abrogation of CCR2 and CCL2 in monocytes/macrophages reduces inflammatory tissue damage in the heart.<sup>25,41</sup> The expression of monocyte/macrophage-attracting cytokines, as well as their receptor molecules, dropped significantly in the inflamed hearts from triple-ip<sup>-/-</sup>, LMP2<sup>-/-</sup>, and LMP7<sup>-/-</sup> mice during Tnl-AM. As shown by reduced infiltration of myeloid immune cells under the influence of ONX 0914, we propose that in mice lacking significant immunoproteasome function, suppressed chemokine production by monocytes is another beneficial aspect mediating protection from cardiac inflammation. Infiltrating monocytes/macrophages are a major source of proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in the inflamed mouse heart.<sup>42</sup> Consistent with previous reports,<sup>15,21,22</sup> production of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  was suppressed during Tnl-AM in immunoproteasome-deficient mice

**Figure 7 Continued.** Red indicates the fraction of genes significantly upregulated in the patient sample in comparison to 2 controls, with the *P* value corrected for multiple testing ( $q < 0.05$ ) and absolute log<sub>2</sub> fold change  $> 0.5$ . **D**, For the autoimmunity gene set, receiver operator characteristic (ROC) is shown. Of 28 genes from the autoimmunity gene set, 17 were present in the analyzed data. Vertical axis and the gray bar next to the curve represents the list of genes ordered by the *P* value of the comparison of cardiac gene transcription between patient 2 and controls. Horizontal dashes indicate genes, which were included in the autoimmunity gene set and their position in the list of transcripts. Horizontal axis shows the fraction of the genes in the gene set. AUC represents the enrichment strength (effect size for the gene set enrichment). Evidence plots for all other gene modules are shown in Figure V in the Data Supplement. **E**, Immunoblot with the 50mer Tnl peptide harboring the immunogenic Tnl epitope VDKVDEERYDVEAKVTKN (lane 1) and human Tnl (lane 2) as antigen stained by Ponceau S stain (left image). Alternatively, for the same immunoblot, serum from patient 2 was used as a primary antibody, and antigen-bound serum antibodies were visualized by chemiluminescence detection of bound antihuman secondary antibody (right image). \*The 50mer Tnl peptide harboring the immunogenic Tnl epitope VDKVDEERYDVEAKVTKN was loaded in lane 1. adj, indicates adjusted; BAFF, B-cell activating factor; CXCL2, C-X-C motif chemokine ligand; MHC, major histocompatibility complex; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; pept, peptide; ROR- $\gamma$ t, RAR-related orphan receptor C- $\gamma$ t; and TLR, Toll-like receptor.





**Figure 8. The multifaceted function of the immunoproteasome in cardiac autoimmune-related disease.**

**A**, On activation, T cells express checkpoint molecules such as PD-1 (programmed cell death protein 1). PD-1 limits potentially aberrant effector function of self-reactive T cells by increasing the threshold needed for activation.<sup>1</sup> **B**, Cancer immunotherapy with inhibitors of the PD-1 pathway reactivates T cells, occasionally with adverse effects on the heart.<sup>4,7,32</sup> In 2 cases of immune checkpoint inhibitor (ICI)-related myocarditis, high cardiac expression of the immunoproteasome, cardiac immunoglobulin G (IgG) deposits, evidence for TnI (troponin I)-directed autoimmunity, and Th17 immunophenotype demonstrate heart-specific autoimmunity. Experimental data from animal models showing myocarditis or dilated cardiomyopathy through the generation of cardiac autoantibodies (TnI, myosin) in PD-1-deficient mice by the Honjo group<sup>8,9</sup> indicate an autoimmune etiology of ICI-related myocarditis in some patients. **C**, In mice, an immunogenic TnI peptide can trigger myocarditis,<sup>11,24</sup> and this TnI peptide was found to be immunogenic in a patient with ICI-related myocarditis. During the onset of cardiac autoimmunity in mice, the immunoproteasome controls CD4<sup>+</sup> T-cell activation and expression of checkpoint molecules and stimulates the production of chemotactic and proinflammatory cytokines (eg, in monocytes), the later governing CD4<sup>+</sup> T-cell differentiation into Th17 and Th1 cells.<sup>27</sup> Subsequently, inflammatory monocytes migrate into the heart, resulting in tissue damage with functional impairment. **D**, Inhibition of the immunoproteasome by ONX 0914 mitigates the autoimmune-related inflammatory response in TnI myocarditis in mice and minimizes heart muscle damage, leading to improved cardiac performance. <sup>§</sup>Findings of this study. ab indicates antibody; APC, antigen-presenting cell; TCR, T-cell receptor; Th1, CD4<sup>+</sup> Th1 cell; Th17, CD4<sup>+</sup> Th17 cell; and Treg, CD4<sup>+</sup> regulatory T cell.

and in TLR2-, TLR7/8-, and TLR4-activated human monocytes lacking immunoproteasome activity. Local secretion of the cytokines IL-1 and TNF by infiltrating inflammatory cells in the heart promotes the induction of autoimmune disease,<sup>43</sup> and there is evidence that high IL-6, IL-1 $\beta$ , or TNF- $\alpha$  production can contribute to cardiodepressive pathology.<sup>27,44</sup> Therefore, lower cytokine production in mice lacking immunoproteasome activity is of high biological relevance for the development of heart failure, and is consistent with a low degree of fibrosis and improved cardiac performance in LMP7<sup>-/-</sup> or ONX 0914-treated mice during TnI-AM.

### Immunoproteasome Inhibitors as a Strategy for Autoimmune-Related Myocarditis

Bortezomib and carfilzomib, which are licensed for the treatment of multiple myeloma,<sup>18</sup> primarily target the highly abundant  $\beta 5$  subunit of the cardiac proteasome complex,<sup>45</sup> and thereby disrupt protein homeostasis in cardiomyocytes, resulting in cell death,<sup>46</sup> thus constituting a risk for the development of cardiac dysfunction. Compounds selectively targeting the immunoproteasome may provide a useful alternative strategy regarding the maintenance of cardiac proteostasis. ONX 0914

injected into mice before or after immunization with the TnI peptide at a dose blocking both LMP7 and LMP2<sup>15</sup> was successful at preventing the development of TnI-AM, verifying the role of the immunoproteasome for activation and expansion of self-reactive T cells. A previous study illustrated the beneficial outcome of the immunoproteasome inhibitor ONX 0914 during severe virus-induced myocarditis, revealing no adverse effect on the formation of an antiviral adaptive and memory immune response.<sup>22</sup> The immunoproteasome has a minor influence on the control of cardiotropic pathogens,<sup>22,47,48</sup> and this is consistent with reports for other pathogens.<sup>36</sup> Another putative concern is the degree of subunit selectivity after long-term application of compounds that, like ONX 0914, bind irreversibly to subunits of the immunoproteasome.<sup>15</sup> If long-term treatment with an immunoproteasome inhibitor is found to be necessary, the use of a reversible immunoproteasome inhibitor with improved subunit selectivity would be preferable.<sup>35</sup>

## Heart-Specific Immune Responses in ICI-Related Myocarditis

Our data unequivocally demonstrate that immunoproteasome inhibitors in a mouse model attenuate autoimmune-related myocarditis. We need further research to define the role of immunoproteasome-dependent proteolysis in human patients with cancer with ICI-related myocarditis. Nevertheless, on the basis of our experimental mouse data, immunoproteasome inhibitors could be suitable principally for the treatment of patients with ICI-related autoimmune myocarditis who have evidence of heart-specific autoimmunity. In 2 patients with ICI-related myocarditis, we showed the activation of heart-specific autoimmune responses. Similar to autoimmune-related myocarditis in mice, we found elevated cardiac immunoproteasome expression and a Th17 immunophenotype. Moreover, in the patient with cancer with ICI-related myocarditis, in whom we conducted RNA sequencing of endomyocardial biopsies, we found significant enrichment of a known gene set that compromised, among other inflammatory markers, hallmark genes of the T- and B-cell response, known to be relevant for experimental autoimmune myocarditis in mice. Preclinical data showing myocarditis or dilated cardiomyopathy through the generation of cardiac autoantibodies (TnI or myosin) in PD-1-deficient mice<sup>8,9</sup> provide further supportive evidence for the autoimmune etiology of ICI-related myocarditis. In our cases of ICI-related myocarditis, we detected IgG deposits in cardiac inflammatory foci, which corresponds to IgG deposits surrounding cardiomyocytes in PD-1<sup>-/-</sup> mouse hearts,<sup>8</sup> later identified to be specific for TnI.<sup>9</sup> Somewhat in contrast with our findings, in 2 previously reported cases of ICI-related myocarditis, such IgG deposits were absent. Nevertheless, in the same patients, common T-cell receptor sequences in infiltrates from the

heart and tumor raise the possibility for heart-specific autoimmunity in these cases as well.<sup>4</sup> Further supporting evidence for heart-specific immunity in ICI-related myocarditis comes from another PD-1 inhibitor-related myocarditis case with detection of preexisting heart-directed autoimmunity.<sup>49</sup> It is noteworthy in this context that, because of the loss of inhibition by PD-1 molecules, effector T cells can be reactivated by ICI.<sup>2</sup> The detection of autoimmune activity against the immunogenic TnI peptide in a case of ICI-related myocarditis, as shown here, provides additional proof for heart-specific autoimmunity, being active or activated at least in this case of ICI-related myocarditis. Together with shared inflammatory transcriptome signatures in ICI-related myocarditis and experimental TnI-AM, this validates the TnI-AM mouse model by showing it reflects human pathology. In the presented case of ICI-related myocarditis, TnI-directed autoantibodies indeed targeted the same immunogenic epitope that we used to trigger autoimmune myocarditis in mice.<sup>24</sup> Although preclinical work and experimental evidence define a link among the physiologic role of immunoproteasome function, PD-1/PD-1L expression, TnI-directed cardiac autoimmunity, and ICI-related myocarditis, further studies should combine murine cancer models with ICI therapy to elucidate the proposed autoimmune pathogenesis of ICI-related myocarditis in a more physiologically relevant context.

The immunoproteasome regulates processes that balance autoimmune responses in the heart. We have shown that immunoproteasome inhibitors can be used therapeutically in a mouse model of TnI-directed cardiac autoimmunity and thus show the potential for further exploration in models of ICI-related myocarditis and potentially also in patients with evidence of heart-specific autoimmunity.

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## Disclosures

None.

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